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Molecular Assays for Detecting *Aphanomyces invadans* in Ulcerative Mycotic Fish Lesions

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The pathogenic oomycete *Aphanomyces invadans* is the primary etiological agent in ulcerative mycosis, an ulcerative skin disease caused by a fungus-like agent of wild and cultured fish. We developed sensitive PCR and fluorescent peptide nucleic acid in situ hybridization (FISH) assays to detect *A. invadans*. Laboratory-challenged killifish (*Fundulus heteroclitus*) were first tested to optimize and validate the assays. Skin ulcers of Atlantic menhaden (*Brevoortia tyrannus*) from populations found in the Pamlico and Neuse River estuaries in North Carolina were then surveyed. Results from both assays indicated that all of the lesioned menhaden ($n = 50$) collected in September 2004 were positive for *A. invadans*. Neither the FISH assay nor the PCR assay cross-reacted with other closely related oomycetes. These results provided strong evidence that *A. invadans* is the primary oomycete pathogen in ulcerative mycosis and demonstrated the utility of the assays. The FISH assay is the first molecular assay to provide unambiguous visual confirmation that hyphae in the ulcerated lesions were exclusively *A. invadans*.

Since the early 1980s, estuarine fish, primarily Atlantic menhaden (*Brevoortia tyrannus*), along the east coast of the United States have experienced seasonal epidemics of deep, aggressive skin ulcers known as ulcerative mycosis (UM) (13, 33). UM has been reported regularly in the spring and fall from the St. Johns River in Florida, the Neuse and Pamlico Rivers in North Carolina, and some tributaries of the Chesapeake Bay (1, 4, 17, 35). Pathological surveys of skin ulcers revealed that most contained oomycetes (filamentous protists that physically resemble fungi) (2, 24, 34). Specialized culture techniques were developed to isolate and identify these oomycetes (3, 13, 46). A number of species belonging to genera as diverse as *Aphanomyces*, *Saprolegnia*, and *Achlya* were recovered (4, 12, 40). When tested, only one species, *Aphanomyces invadans* (synonymous with *Aphanomyces piscicida*), was pathogenic (3, 22, 23). This species occurs worldwide and infects both wild and cultured fish, often leading to mass fish mortality (4, 9, 27, 40). It was originally documented as the cause of mycotic granulomatosis of ayu (*Plecoglossus altivelis altivelis*) in Japan (18) and more recently as the cause of epizootic ulcerative syndrome or red spot of striped snakehead (*Channa striata*) and striped mullet (*Mugil cephalus*) from southern Asia and Australia (9, 15, 21, 30, 40). These skin diseases (mycotic granulomatosis, epizootic ulcerative syndrome, red spot, and UM) are clinically identical and occur in either freshwater or estuarine fish.

Routine identification of oomycetes directly from UM lesions is difficult because the species present in the lesions do not form the specialized reproductive structures required to differentiate one species from another. Thus, oomycetes must be cultured from the lesions and the reproductive structures must be formed before species identifications can be made. The culture recovery rate of oomycete pathogens typically is very low, often $\leq$10% (12, 13). Other microbes that are commonly present at high levels in UM lesions, especially bacteria, outcompete slow-growing species such as *A. invadans* in culture (40). Even if successfully cultured, oomycetes often fail to produce the sexual structures needed for identification to species. These cultural and identification problems have restricted our understanding of the biology, pathogenesis, and epidemiology of this emerging aquatic disease and have limited the identification of this pathogen to a relatively small number of fish with UM. At present, only two *A. invadans* isolates have been reported in culture from the United States (3, 12).

Our objectives in this study were to develop species-specific PCR and in situ hybridization assays to rapidly detect and identify *A. invadans* and to use the assays to screen populations of infected fish. In recent years, other PCR assays have been developed to detect *A. invadans* from formalin-preserved fish samples and from artificially infected fish (3, 38). These assays, however, have not been used for large-scale screening of fish populations. They also lack the visual capacity of fluorescent peptide nucleic acid in situ hybridization (FISH), which can be

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TABLE 1. Oomycete isolates used for PCR and in situ hybridization assay optimizations

Genus and species	Isolate	Source
Aphanomyces		
A. invadans	WIC	*Brevoortia tyrannus*; Great Wicomico River, Va.
A. invadans		*Brevoortia tyrannus*; Pamlico River, N.C.
A. invadans		*Brevoortia tyrannus*; St. Johns River, Fla.
A. astaci[a]	FDL 457	*Austropotamobius pallipes*; Herefordshire, United Kingdom
A. frigidophilus	ATCC 204464	*Onchorhynchus masou*; Japan
Aphanomyces sp.	ATCC 62427	*Brevoortia tyrannus*; Pamlico River, N.C.
Aphanomyces sp.	ATCC 58381	*Xiphinema americanum*; Pa.
Saprolegnia		
S. ferax	ATCC 36051	Lake water; Canada
S. diclina	ATCC 200018	*Oncorhynchus mykiss*; Tokyo, Japan
S. parasitica	ATCC 200015	*Oncorhynchus kisutch*; Miyagi, Japan
Achlya		
A. americana	ATCC 22599	Dead beetle floating on puddle of water; Md.
A. bisexualis		*Mugil cephalus*; Caloosahatchee, Fla.

[a] Data are from reference 36.

TABLE 2. Oligonucleotide primers used in this study to amplify and sequence oomycete SSU, ITS1, 5.8S, ITS2, and the first approximately 60 bp of the LSU

Primer name	Sequence (5′–3′)	Location (bp)
PCR assay primers		
AINVAD-2F	TCATTGTGAGTGAAACGGTG	1674–1693[a]
AINVAD-ITSR1	GGCTAAGGTTTCAGTATGTAG	1888–1908[a]
Forward sequencing primers		
OMYC18SF	GTCTCAAAGATTAAGCCATGC	1–21[a]
OMYC200F	ACGGGTAGCATTTATTAGATT	166–186[a]
OMYC435F	GTGACAATAAATAACAATGC	427–446[a]
OMYC935F	ATCGAAGATGATTAGATACC	957–976[a]
OMYC1700F	TGAATGACTCGGTGAGAGAAATTGGG	1632–1655[a]
OMYCF	AGGTGAACCTGCGGAAGG	1751–1768[a]
Reverse sequencing primers		
OMYCR	TAGCTTAAGTTCAGCGGGT	2477–2495[b]
OMYC1900R	CGGACACTGATACAGACATACTTC	2070–2093[a]
OMYC700R	ATTATTCCATGCTAATGTATTC	756–777[a]
OMYC550R	TTGGAGCTGGAATTACCG	536–553[a]

[a] Primer site locations correspond to GenBank accession number AF396684.
[b] Primer site location corresponds to GenBank accession number AF396683.

used to distinguish *A. invadans* from other oomycete species that may be present in UM lesions.

MATERIALS AND METHODS

Collection and storage of ulcerated fish. Atlantic menhaden were collected (September 2004) by cast net from the Pamlico (35°28′41.9988″, 76°49′0.9984″) and Neuse (34°58′15.7801″, 76°55′45.2639″) Rivers, North Carolina. Fifty arbitrarily selected fish, exhibiting typical UM skin ulcers (34), were placed in plastic bags and transported to the laboratory on ice. Control fish without lesions were also collected. Specimens that were not necropsied and assayed immediately were frozen whole at −80°C.

Oomycete isolates and sporulation. Oomycete isolates used to validate the molecular assays (Table 1) were either obtained from the American Type Culture Collection (ATCC) or cultured from lesioned fish tissue by standard methods (4, 46). Isolates from Florida were shipped as needed on agar slants to the National Oceanographic and Atmospheric Administration Center for Coastal Fisheries and Habitat Research Laboratory, Beaufort, NC. All isolates were maintained at 24°C on Griffin's glucose-yeast (GY) broth or Griffin's GY agar (16) and were transferred to fresh medium every 21 days. Oomycete cultures were also sporulated (23) to evaluate the ability of the FISH assay to detect zoospore stages of *A. invadans*.

Experimental infections. Killifish (*Fundulus heteroclitus*) were infected artificially with *A. invadans* to provide material for validating and optimizing the PCR and FISH assays. Thirty healthy adult fish obtained from a *Spartina* marsh adjacent to the Center for Coastal Fisheries and Habitat Research Laboratory were divided into three treatment groups (10 fish per group). The fish were acclimated to 21°C and a salinity of 4 practical salinity units for 2 days in separate 38-liter aquaria. Fish were fed flake food daily. Water quality was monitored weekly, and water changes were made daily with natural seawater from Gallant Channel, North Carolina, diluted with deionized water to a salinity of 4 practical salinity units.

Griffin's GY broth (25 ml) was inoculated with 5-mm agar plugs of 4-day-old *A. invadans* or *Aphanomyces* sp. 84-1240 cultures cut out with a sterilized cork borer. *Aphanomyces* sp. 84-1240 (ATCC 62427) is a nonpathogenic strain (13, 23) that served as a negative control. The broth cultures were grown in the dark for 3 days in sterile 50-ml plastic, conical, centrifuge tubes at 24°C. Cultures were rinsed three times with 25 ml of sterile, deionized water and were then transferred to a 100-mm petri dish. Five-millimeter lengths of actively growing hyphal tips ($n = \sim 50$) were cut aseptically from a mycelial mat with a sterile scalpel blade. The hyphal tips were macerated with the scalpel blade, suspended in 1 ml of sterile deionized water, and drawn into a 22-gauge needle for injection into killifish.

All fish were individually anesthetized with 150 mg · liter^{-1} of 3-aminobenzoic acid ethyl ester (MS 222) in aquarium water prior to injection. The first group was injected with 0.1 ml of *A. invadans* hyphal suspension; the second group was injected with 0.1 ml of *Aphanomyces* sp. 84-1240. The third group, serving as a sham injection control, was injected with 0.1 ml of sterile deionized water. All fish were injected intramuscularly near the base of the dorsal fin and returned to the aquaria. The fish were fed and monitored daily for 2 weeks for the development of lesions. At the end of the experiment, fish were euthanized with >500 mg · liter^{-1} of MS 222; lesioned tissues (skin and muscle) were removed aseptically by standard necropsy procedures. Tissues that were not used immediately were stored at −80°C until needed.

DNA extraction, PCR, and sequencing protocol. The ClustalX algorithm was used to align rRNA gene sequences, available from GenBank, of *Aphanomyces* spp., *Saprolegnia* spp., and *Achlya* spp. (accession numbers AF396683, AJ238655, M32705 J02951, AJ238662, and AJ238656). Oligonucleotide primers (OMYC 18SF and OMYCR) were designed for the amplification of the entire small subunit (SSU), the first internal transcribed spacer region (ITS1), the 5.8S subunit (5.8S), the second internal transcribed spacer region (ITS2), and the first 42 bp of the large subunit (LSU) rRNA genes.

DNA was extracted from an *A. invadans* culture originally isolated from an Atlantic menhaden from the Great Wicomico River, Virginia. Actively growing colonies in GY broth were transferred to sterile 100-mm petri dishes, and the GY broth was decanted from the dish. Hyphal tips were excised with a sterile scalpel blade (total of ~250 mg) and transferred to a microcentrifuge tube for DNA extraction with an UltraClean soil DNA extraction kit (Mo Bio Laboratories, Inc., Solana Beach, Calif.), following the manufacturer's protocol. The amplification reaction mixture contained 100 mM Tris-HCl (pH 8.3), 15 mM $MgCl_2$, 750 mM KCl, 25 pM each primer, 2.5 mM each deoxynucleoside triphosphate, 0.5 U of Platinum *Taq* DNA polymerase (Invitrogen Life Technologies, Rockville, Md.), and 20 ng of genomic DNA in a total reaction volume of 50 μl. The DNA was amplified in a Robocycler (Stratagene, La Jolla, Calif.) under the following cycling conditions: 2 min at 95°C; 35 cycles, each consisting of 30 s at 95°C, 45 s at 56°C, 2.5 min at 72°C; and a final extension of 5 min at 72°C.

A 5-μl aliquot of each PCR was evaluated by agarose gel electrophoresis. PCRs containing specific products were purified with the QIAquick PCR purification kit (QIAGEN, Valencia, Calif.), quantified spectrophotometrically, and sequenced with an ABI377 DNA sequencer with the Deoxy Terminator Cycle sequencing kit (Applied Biosystems-ABI, Foster City, Calif.). Conserved rRNA gene regions were evaluated for internal sequencing primers (Table 2). DNA templates were sequenced in both directions with the sequencing primers listed in Table 2. The resulting SSU-to-5′ LSU rRNA gene sequence for *A. invadans* was assembled by using the Vector NTI program (Informax, Inc., Bethesda, Md.).

Development of FISH assays. We used peptide nucleic acid (PNA) probes, which are neutrally charged synthetic molecules that mimic the structure of both

DNA and RNA (5, 31, 47) but have a higher melting temperature per base pair than do conventional RNA or DNA probes. The *A. invadans* SSU rRNA gene sequence obtained in this study was aligned with the SSU rRNA gene GenBank sequences listed above to design the species-specific probe. A unique hybridization site was identified (bp 621 to 635; AF396684), and a species-specific PNA probe for *A. invadans* with a 5′ fluorescein (FLU) conjugate was manufactured (5′-FLU-GTACTGACATTTCGT-3′, designated Ainv-FLU3; Applied Biosystems, Bedford, Mass.).

In situ hybridization procedures were adapted from those developed for dinoflagellates (28, 29). Cultured hyphae, cultured zoospores, and infected tissues were fixed to preserve the integrity of the cells and to prevent rRNA degradation. For cultured hyphae, the GY broth was decanted from culture tubes and the hyphae were rinsed twice for 10 min each time with 20 ml of sterile, deionized water in the culture tubes. After the second rinse, 25 ml of an ethanol-saline fixative was added to the tube to preserve the hyphae. The ethanol-saline fixative contained 44 ml of 95% ethanol, 10 ml of deionized H_2O, and 6 ml of 25× SET buffer (3.75 M NaCl, 25 mM EDTA, 0.5 M Tris HCl [pH 7.8]). Cultured hyphae were fixed for at least 30 min prior to hybridization.

Fixation of zoospore cultures was similar, except that the zoospores were allowed to settle for 15 min prior to fixation. The culture medium was removed with a pipette, leaving 5 ml of a concentrated zoospore suspension. Thirty milliliters of ethanol saline was used to fix the zoospores for at least 30 min before hybridization was initiated.

Fixation and permeabilization of *A. invadans*-infected fish tissue were optimized by using experimentally infected *F. heteroclitus* and lesioned material from naturally infected Atlantic menhaden. To minimize RNA degradation, fixation and hybridization procedures were carried out as soon as possible after the fish were collected. Tissues were also removed from nonlesioned fish and assayed to check for cross-reactivity and to serve as negative controls. Tissue (~20 mg) was dissected from the periphery of the lesions with sterile scalpel blades and placed in individual wells of a 24-well microtiter dish. One milliliter of ethanol-saline fixative containing 3% polyoxyethylenesorbitan monolaurate (Tween 20) was added to enhance tissue permeabilization. The microtiter dish was gently agitated at room temperature on an orbital shaker (30 rpm) for 1.5 h.

FISH of cultured oomycete hyphae was carried out in the 24-well microtiter dishes. Small pieces (each, ~5 mm^3) of ethanol-saline-preserved hyphae were washed (twice for 15 min each time) with 0.5-ml aliquots of hybridization buffer [5× SET, 0.1% (vol/vol) Igepal-CA630 (Sigma, St. Louis, Mo.), and 25 $\mu g \cdot ml^{-1}$ poly(A) (Sigma)]. The hybridization buffer was removed, and the hyphae were resuspended in 0.5 ml of hybridization buffer containing 20 nM of the Ainv-FLU3 probe. Hyphae in the "no-probe" control wells were resuspended in 0.5 ml of hybridization buffer only. All samples were incubated at 60°C for 1 h in the dark. Following incubation, the probe solution was removed, and the samples were rinsed with 1 ml of prewarmed (60°C) 5× SET buffer.

A. invadans zoospores were prepared for FISH by vacuum filtration of 3-ml aliquots of preserved zoospore cultures through 13-mm by 0.2-μm polycarbonate membrane filters (28). The zoospores were resuspended with hybridization buffer, probed, and rinsed in the filter apparatus with the reagents under the conditions described above.

For FISH of lesions, the fixed tissues were rinsed (twice for 15 min each time) with 0.5 ml of hybridization buffer containing 3% Tween 20. The hybridization buffer was removed, and the tissues were resuspended in 0.5 ml of hybridization buffer containing 3% Tween 20 and 100 nM Ainv-FLU3 probe. "No-probe" control specimens were incubated with 0.5 ml of hybridization buffer–3% Tween 20. All tissues were incubated at 60°C for 1 h in the dark. Following incubation, the tissues were rinsed twice with 1 ml of prewarmed (60°C) 5× SET buffer containing 3% Tween 20 to remove residual probe.

All specimens were mounted onto poly-L-lysine-coated microscope slides. One drop of Slowfade Light Antifade solution from Molecular Probes, Inc. (Eugene, Oreg.), was placed on the specimens and then overlaid with a coverslip. Analyses were performed with light and epifluorescence microscopy. Camera and microscope settings for epifluorescent analysis were held constant so that comparative analyses of relative fluorescence intensity could be made between probed and nonprobed specimens.

Each FISH assay included a positive, a negative, and a "no-probe" control. The positive control consisted of a PNA probe (EuUni-1, FLU-ACCAGACTT GCCCTCC; bp 509 to 524; AF396684) that hybridizes to the SSU rRNA of all eukaryotes. This probe was used to confirm that the PNA probes could penetrate all of the cell types and that the rRNA had not degraded. The negative control consisted of a PNA probe specific for the SSU rRNA of the heterotrophic dinoflagellate *Pfiesteria piscicida* (PpiscFLU-1, 5′-FLU-GAAAGTGATATGGTA-3′; bp 253 to 268; AF396684). This probe was added to samples at concentrations equal to or higher than that used for the Ainv-FLU3 probe to determine whether positive signals were due to nonspecific hybridization. The no-probe control assessed native autofluorescence in fixed cells and tissues. The tissue samples were then analyzed by epifluorescent microscopy.

We observed in initial experiments that a single freeze-thawing cycle of frozen tissue resulted in significant loss of RNA and a decrease in the fluorescent signal from the hybridized cells. Therefore, FISH assays were always performed the first time a sample was thawed. In contrast, several cycles of freezing and thawing did not affect the sensitivity of the PCR assays.

Microscopy and image acquisition. High-resolution photomicroscopy of the in situ hybridization experiments was conducted with an Orca II digital camera (Hamamatsu Photonics, Hamamatsu, Japan) and a Sony (Tokyo, Japan) 3CCD color video camera. The cameras were mounted on a Nikon (Tokyo, Japan) TE300 Eclipse inverted microscope. The cameras were interfaced with a desktop computer driven by Metamorph image acquisition and analysis software from Universal Imaging (Downingtown, Pa.). Differential interference contrast and epifluorescence microscopy (fluorescein isothiocyanate excitation (λ_{ex}) and emission (λ_{em}) settings were as follows: λ_{ex} = 460 to 500 nm; λ_{em} = 510 nm; dichroic long-pass beam splitter = 505 nm) were used to visualize the specimens.

Species-specific PCR assay development. The SSU 5′ LSU rRNA gene sequence for *A. invadans* obtained in this study was aligned by using the ClustalX program with sequences from other related *Aphanomyces* spp., *Pythium* spp., and *Achlya* spp. sequences available from GenBank (accession numbers AF396683, AF396684, AF330186, AF452162, AF218162, AF218159, and AF218158). The oomycete ITS regions contained a larger number of unique species-specific primer sites relative to the flanking SSU, 5.8S, and LSU genes. The PCR assay utilized a species-specific forward primer site located near the 3′ end of the SSU gene and a species-specific reverse primer site located in the ITS1 region. PCR amplification using the *A. invadans* primers Ainvad-2F (5′-TCATTGTGAGTG AAACGGTG-3′) and Ainvad-ITSR1 (5′-GGCTAAGGTTTCAGTATGTAG-3′) generated a 234-bp product.

PCRs contained 25 pM each primer, 2.5 mM each deoxynucleoside triphosphate, 0.5 U of Platinum *Taq* DNA polymerase (Invitrogen), and 20 ng of genomic DNA for a total volume of 50 μl. Amplifications were carried out in a Robocycler with the following program: 2 min at 95°C; 35 cycles, each consisting of 20 s at 95°C, 30 s at 54°C, and 45 s at 72°C; and a final extension of 5 min at 72°C. An aliquot (5 μl) from each amplification was analyzed by agarose gel electrophoresis. The size of the PCR product was estimated by comparisons with a 100- or 123-bp molecular weight ladder (Promega, Madison, Wis., and Roche, Basel, Switzerland, respectively).

PCR assay of ulcerated tissues. The primer pairs were tested for cross-reactivity to DNA extracted from other oomycetes (Table 1) and from nonlesioned fish (*B. tyrannus* and *F. heteroclitus*). DNA was also extracted from ulcerated tissues (10 mg) removed from 50 Atlantic menhaden with a DNeasy Tissue kit following the manufacturer's protocol (QIAGEN). DNA (50 ng) from each lesioned sample was added to the PCR mixtures and amplified as described above. Each PCR assay included a positive control, a negative control, a blank DNA extraction control, and a PCR inhibition control. The positive control incorporated 30 ng of *A. invadans* genomic DNA in the reaction mixture. The negative control substituted 1× PCR buffer for DNA in a subset of reaction mixtures to confirm that the reagents were not contaminated. The blank extraction controls were included to rule out possible cross-contamination during the DNA extraction process. The inhibition control consisted of spiking 30 ng of *A. invadans* genomic DNA into an arbitrarily chosen ulcerated menhaden DNA sample. The inhibition control ensured that negative PCRs were not due to PCR inhibition but to the absence of *A. invadans* DNA.

Nucleotide sequence accession number. The SSU-to-5′ LSU rRNA gene sequence for *A. invadans* was submitted to GenBank and assigned accession number AF396684.

RESULTS

The *A. invadans* FISH probe was first tested for species specificity and cross-reactivity by hybridization to three strains of *A. invadans* and to nine related oomycetes (*Aphanomyces* spp., *Saprolgenia* spp., and *Achyla* spp.) (Table 1). No cross-reactivity occurred (Fig. 1). The probe's ability to hybridize *A. invadans* secondary zoospores was also confirmed (Fig. 2). Laboratory-challenged killifish (*F. heteroclitus*) injected with *A. invadans* hyphae developed lesions and hyphal infection throughout much of the body. Microscopic observation showed that hyphae embed-

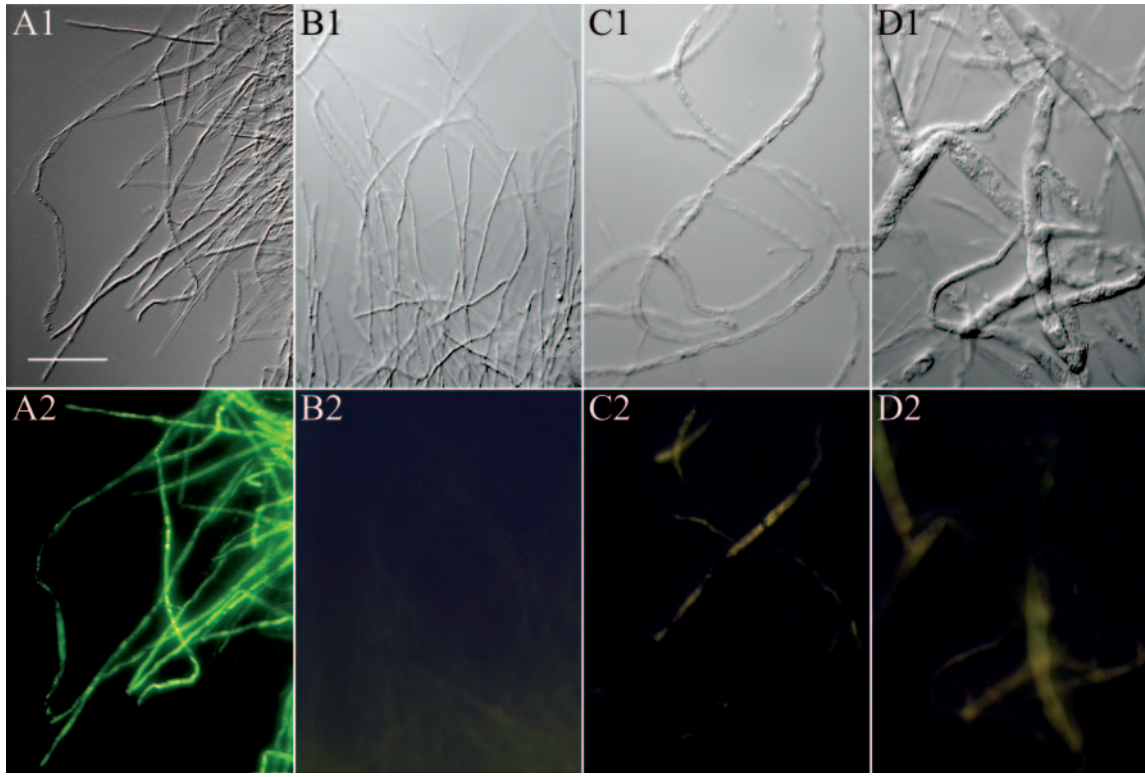

FIG. 1. The *Aphanomyces invadans* FISH probe (Ainv-FLU3) was tested for cross-reactivity by hybridization to other closely related oomycetes. Light micrographs (top) and epifluorescent micrographs (bottom) are shown of *Aphanomyces invadans* (A), *Aphanomyces astaci* (B), *Saprolegnia parasitica* (C), and *Achlya americana* (D). Note the green fluorescence of the probe (A2) versus the orange autofluorescence of *S. parasitica* (C2) and *A. americana* (D2) hyphae. The other oomycetes listed in Table 1 were also hybridization negative (data not shown). Scale bar, 20 μm.

ded in the infected tissues exhibited the same aseptate branching pattern as was observed for *A. invadans* grown in culture. Killifish receiving the sham injections or injected with the nonpathogenic *Aphanomyces* sp. 84-1240 had a 100% survival rate and did not show any signs of infection or lesion formation. Samples of *A. invadans*-infected killifish tissue were used to empirically determine optimal hybridization conditions for the FISH probes.

Validation of *A. invadans* FISH assay. Fifty Atlantic menhaden exhibiting typical UM ulcers were assayed with the FISH probes. A universal eukaryote probe was used as the positive control, ensuring that the hyphae were permeabilized and that the rRNA was intact (Fig. 3A). The *A. invadans*-specific probe successfully hybridized to hyphae that typically spread throughout the tissue samples (Fig. 3B). The negative control probe was specific for the dinoflagellate *Pfiesteria piscicida* and was used to assess nonspecific probe hybridization, which did not occur in any of the experiments (Fig. 3C). A "no-probe" control assessed autofluorescence of the menhaden tissue and of the hyphae (Fig. 3D). All of the UM menhaden were positive for *A. invadans* by the FISH assay. Tissues assayed from nonlesioned fish were negative.

Validation of *A. invadans* PCR assay. Experiments testing the potential cross-reactivity of the *A. invadans*-specific PCR assay were performed with genomic DNA of other oomycete species (Table 1). These experiments confirmed that the *A. invadans* PCR assay was species specific and did not amplify DNA from related oomycete species (Fig. 4A). DNA was ex-

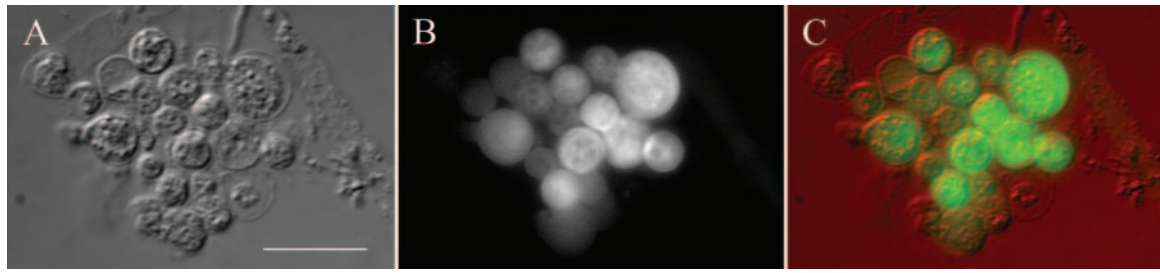

FIG. 2. Hybridization of the Ainv-FLU3 probe to *Aphanomyces invadans* secondary zoospores. A light micrograph (A) and epifluorescent micrograph (B) are shown. (C) A falsely colored epifluorescent image is superimposed on the light micrograph. Scale bar, 10 μm.

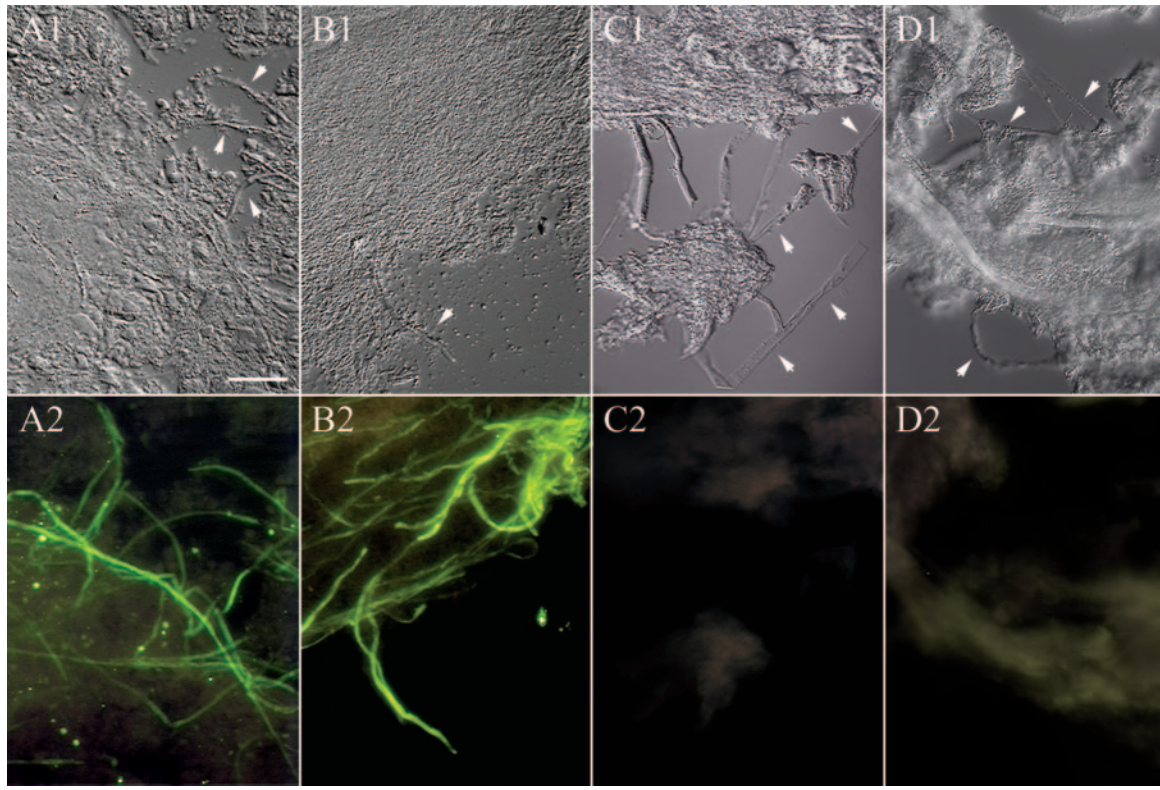

FIG. 3. *Aphanomyces invadans* FISH assay of UM-infected Atlantic menhaden. Light micrographs (top) and epifluorescent micrographs (bottom) are shown. (A) Hybridization of the positive control probe EuUni-1. (B) Hybridization of the Ainv-FLU3 probe. (C) Hybridization of the negative control probe, PpiscFLU-1. (D) No-probe control. Arrowheads indicate *A. invadans* hyphae. Scale bar, 20 μm.

tracted from the same UM lesions that were screened with the FISH assay and subjected to PCR. *A. invadans* was detected by PCR in every lesioned tissue sample, exactly matching the FISH assay results (Fig. 4B). Tissues from nonlesioned fish were negative by the PCR assay.

DISCUSSION

Ulcerative mycosis is a serious fish disease caused by the pathogenic oomycete (water mold) *A. invadans*. This study describes the development of FISH and PCR assays that can rapidly detect *A. invadans* from naturally infected fish tissues. The specificity of the FISH assay was tested using a panel of related oomycetes, including four other *Aphanomyces* taxa (Fig. 1). After specificity was confirmed, killifish were injected with *A. invadans* hyphae grown in culture. The artificial infections verified the pathogenicity of the *A. invadans* culture and provided material for optimizing the assays. *A. invadans* zoospores were also assayed to show that the probe was capable of hybridizing additional life cycle stages of *A. invadans* (Fig. 2). The FISH protocol utilizes whole-cell and tissue samples and can be rapidly executed without preparing histological sections. When 50 ulcerated Atlantic menhaden collected from the Neuse and Pamlico River estuaries in North Carolina were screened, the lesions from all 50 fish were positive for *A. invadans*. A comparison of light micrographs and FISH images from each of the samples showed that all oomycete hyphae within the ulcerated tissue samples were uniformly *A. invadans* (Fig. 3). The FISH assay further confirmed that the hyphae were not merely surface contaminants but had penetrated deeply past the skin and into the muscle tissue. This work represents the first assay capable of providing rapid, species-specific, visual identification of *A. invadans* hyphae in wild-caught ulcerated fish.

The species specificity of the PCR assay developed in this study was similarly validated using a panel of oomycete cultures (Table 1). The assay was used to survey the same 50 infected fish. Each UM tissue sample tested positive for *A. invadans*. The agreement between the FISH and PCR assays indicates that PCR alone could be used as a reliable diagnostic test for the presence of *A. invadans* infection. In addition to this assay, other *A. invadans* PCR assays are available (3, 26, 38). Blazer et al. (3) used PCR to assay an unspecified number of naturally infected, formalin-preserved fish from 13 UM epidemics to demonstrate the broad geographic range of *A. invadans* extending from the Indian River in Delaware to a farm pond in Louisiana. Lilley et al. (26) tested a PCR assay on cultures of 12 *A. invadans* isolates and 12 other oomycetes to document that their assay was species specific. In a similar study, Phadee et al. (38) used a different PCR assay to screen 20 *A. invadans* isolates and >50 other isolates belonging to the Saprolegniaceae. Phadee et al. (38) also used the assay to detect *A. invadans* in experimentally challenged goldfish and estimated a lower limit of detection for the assay of ~500 fg of DNA. While the above PCR studies have provided valuable information, our work is the first large-scale sampling of a

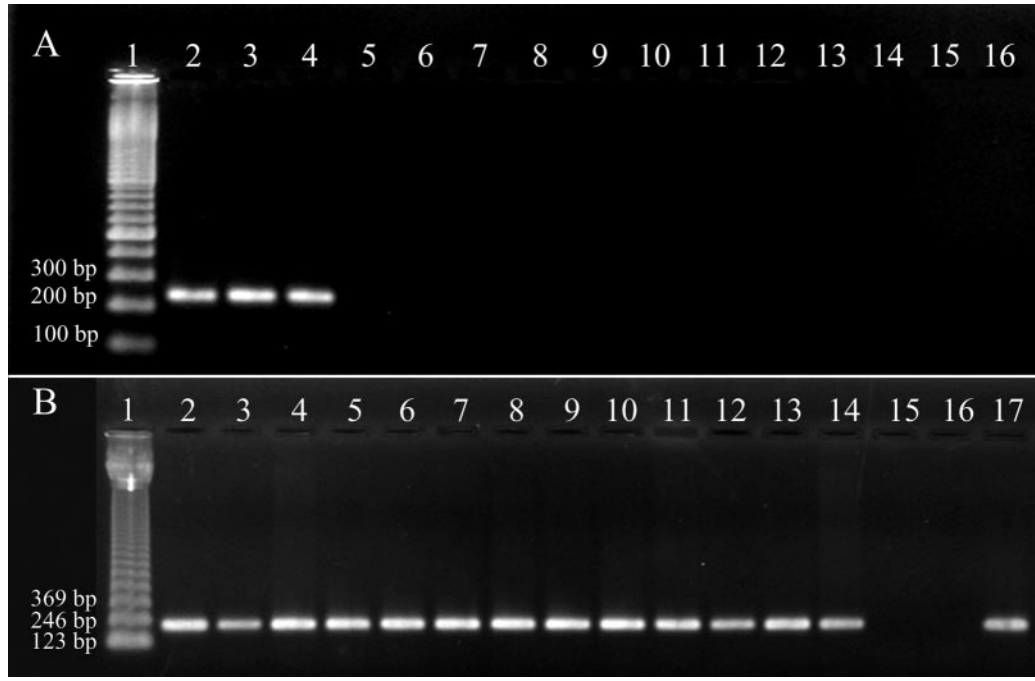

FIG. 4. (A) The *Aphanomyces invadans* PCR primers were tested for cross-reactivity with genomic DNA from three strains of *A. invadans*, nine other related oomycetes, and two fish species. Lane 1, 100-bp ladder; lanes 2 to 4, *A. invadans*; lane 5, *A. astaci*; lane 6, *Aphanomyces frigidophilus*; lane 7, *Aphanomyces* sp. ATCC 62427; lane 8, *Aphanomyces* sp. ATCC 58381; lane 9, *Saprolegnia ferax*; lane 10, *Saprolegnia diclina*; lane 11, *S. parasitica*; lane 12, *Achlya americana*; lane 13, *Achlya bisexualis*; lane 14, *Fundulus heteroclitus*; lane 15, *Brevoortia tyrannus*; lane 16, negative DNA control. (B) *Aphanomyces invadans* PCR assay of ulcerated menhaden. Lane 1, 123-bp ladder; lane 2, *A. invadans*-positive control; lanes 3 to 14, DNA from ulcerated menhaden tissues; lane 15, negative DNA control; lane 16, blank DNA extraction control; lane 17, PCR inhibition control.

population of naturally infected fish and is the first to demonstrate that the oomycete hyphae in the lesions were uniformly *A. invadans*. Unlike the previous studies, our study also included experimental controls that are essential for confirming the specificity and accuracy of a PCR assay. These included a PCR inhibition control to rule out the possibility of false-negative samples and a blank extraction control to identify potential DNA contamination during DNA extraction procedures.

In the past, the etiology of UM in estuaries in North Carolina, the Chesapeake Bay, and Florida has been the subject of great controversy. Initial work in the 1980s indicated that oomycetes were present in the majority of UM lesions (13, 33). Positive species identification of the oomycetes in these lesions was not possible at the time, since the necessary molecular techniques were not available, but it was hypothesized that an *Aphanomyces* species was involved in the etiology of UM (13, 33, 35). In the early to mid-1990s, such lesions were attributed to outbreaks of the putatively toxic zoospore stages of the dinoflagellate *Pfiesteria piscicida* (6–8, 14). The relationship between *Pfiesteria* and fish lesions, however, was based on laboratory studies that did not simulate natural conditions. Those studies were challenged by a number of researchers (10, 11, 41–44), and there is now a large body of research evidence that supports the primary role of *A. invadans* in causing UM. Repeated histological surveys of UM-affected fish have found a high number of broad aseptate hyphae in the ulcers (2, 4, 12, 13, 24, 25, 35). PCR confirmed the presence of *A. invadans* in formalin-preserved ulcers (3), and laboratory exposure studies have shown that *A. invadans* zoospores can induce typical UM lesions (19, 22, 23, 27). In addition, researchers in southern Asia and Australia have identified *A. invadans* as the cause of similar ulcerative lesions in multiple fish species, including striped snakehead (*Channa striata*) and striped mullet (*Mugil cephalus*) from both freshwater and estuarine habitats (9, 15, 21, 30, 40, 45). The collective data from these studies, combined with the data from our study, support the hypothesis that *A. invadans* is the primary infectious etiological agent of UM in estuarine fish in the southeastern United States and many other regions of the world. *A. invadans*, however, should not be considered the sole etiology for ulcerative lesions. Stress, poor nutrition, and certain parasite, bacterial, and viral infections are all capable of producing similar lesions (20, 24, 32, 39).

An important question concerning *A. invadans* pathogenesis that has yet to be answered is how natural infections are initiated. Although hundreds of UM lesions have been examined, no oomycete reproductive structures have been observed (E. J. Noga, unpublished data). This observation implies that although *A. invadans* readily infects fish and often proliferates rapidly, fish are probably a dead end host. Either the infections are transferred directly between fish (less likely) or an alternate source of infection is present in the environment. Laboratory exposure studies suggest that secondary zoospores are the most likely source of UM infection (19, 22, 23). Temperature and salinity preferences, as well as sporulation cues, suggest that *A. invadans* sporulates and proliferates in low-salinity portions of estuaries following runoff events (3, 4, 13, 23, 27, 37). These low-salinity estuaries also serve as nurseries for large populations of juvenile Atlantic menhaden (1), the

most commonly infected fish in the southeastern United States. The PCR and FISH assays developed in this study now make it possible to screen the large numbers of environmental samples needed to identify alternative hosts and sources of inoculum for *A. invadans* infections. Identification of the infection sources and the conditions that promote growth and transmission of this pathogen will help resource managers better predict when lesion events are likely to occur and perhaps develop effective mitigation strategies.

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